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Methanol metabolism in thermotolerant methylotrophic *Bacillus* species

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1. SUMMARY

For a number of years we have tried to isolate versatile methylotrophic bacteria employing the ribulose monophosphate (RuMP) cycle of formaldehyde fixation. Recently this has resulted in the development of techniques for the selective enrichment and isolation in pure culture of *Bacillus* strains able to grow in methanol mineral medium over a temperature range between 35 and 60 °C. At the optimum growth temperatures (50–55 °C), these isolates display doubling times between 40 and 80 min. The metabolism of the strains studied is strictly respiratory. Methanol assimilation is exclusively via the RuMP cycle variants with the fructose bisphosphate (FBP) aldolase cleavage and transketolase (TK)/transaldolase (TA) rearrangement. Whole cells were unable to oxidize formate, and no activities of NAD-(in)dependent formaldehyde and formate dehydrogenases were detected. Formaldehyde oxidation most likely proceeds via the so-called dissimilatory RuMP cycle. The initial oxidation of methanol is catalyzed by an NAD-dependent methanol dehydrogenase present as an abundant protein in all strains. The enzyme from *Bacillus* sp. C1 has been purified and characterized.

2. INTRODUCTION

Only a limited number of Gram-positive methylotrophic bacteria has been isolated in pure culture thus far (Table 1). As a consequence, little is known about the enzymology of one-carbon metabolism in Gram-positive organisms. The available evidence indicates that these organisms are facultative methylotrophs, employing the RuMP cycle of formaldehyde fixation. These organisms are of interest both from a fundamental and an applied point of view. In recent years it has become evident that especially the biochemistry of methanol oxidation in these strains is different from that normally encountered in Gram-negative bacteria. Moreover, Gram-positive non-methylotrophic bacteria have found wide application in the production of fine chemicals such as amino acids. Studies in this direction may be especially rewarding also with methanol-utilizing coryneform, actinomycete and *Bacillus* species that become available. In our experience, these facultative methylotrophs are more amenable to the extensive physiological and genetical manipulation required for strain development than the obligate Gram-negative RuMP cycle methylo-

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Table 1

Some characteristic properties of Gram-positive facultative methylotrophs employing the RuMP cycle of formaldehyde fixation

Organism	Growth substrate	Mode of cleavage	Growth rate (h^{-1})	Temp. opt. (°C)	Ref.
Arthrobacter P1	MA	FBPA	0.25	30	[1]
A. globiformis B-175	MA	FBPA		20	[2]
Brevibacterium fuscum 24	MA	FBPA			[3]
Mycobacterium vaccae 10	Methanol	FBPA			[3]
Mycobacterium gastri	Methanol [a]			37	
Nocardia sp. 239	Methanol	FBPA	0.12	37	[4]
Bacillus PM6	TMA	FBPA	0.80	37	[5]
Bacillus S2A1	TMA	FBPA	0.80	37	[5]
Bacillus sp. C1	Methanol	FBPA	0.50	55	[6]
Bacillus sp. TS4	Methanol	FBPA	0.90	55	[6]

[a] N. Kato, unpublished results. MA, methylamine; TMA, trimethylamine; FBPA, fructose bisphosphate aldolase.

trophs. In this paper recent studies on methanol metabolism in thermotolerant *Bacillus* species are described (Table 1).

3. ISOLATION OF METHYLOTROPHIC *BACILLUS* SPECIES IN PURE CULTURE

The details of the procedures followed have been reported previously [6]. Incubation of pasteurized (or non-pasteurized) material from various sources in methanol mineral medium at temperatures of 50–60 °C resulted in the rapid establishment of mixed cultures of *Bacillus* species. Mixed cultures of endospore-forming methanol-utilizing bacteria have been described before but attempts to obtain pure cultures met with failure [7]. Initially we experienced similar problems. The colonies that were obtained on methanol agar contained many lysed cells and (non-methylo-

Table 2

General properties of methylotrophic *Bacillus* strains

Gram-positive, endo-sporeforming, rod-shaped bacteria	
Dimensions of cells	: 0.5–1.0 μm (width) × 1–5 μm (length)
Metabolism	: strictly respiratory
Growth temperature range	: 35–60 °C
t_{d} on methanol at 50–55 °C	: 40–80 min
pH range for growth	: 5–9
pH optimum for growth	: 7.0–7.5
Molar growth yield	: 16–18 g dry weight/mole methanol
Assimilation pathway	: ribulose monophosphate pathway
Methanol oxidation	: NAD-dependent alcohol dehydrogenase
Methanol tolerance	: >1.5 M
Common growth substrates	: methanol, glucose, maltose, mannitol, pyruvate, nutrient broth
Common nitrogen sources	: ammonia, glutamine
No growth supplements required	: *Bacillus* sp. TS1, AR2, C1
Growth supplements required:	
Bacillus sp. P1	: biotin, thiamine, folic acid
Bacillus sp. TS2	: biotin, vitamin B12, lipoic acid
Bacillus sp. TS4	: vitamin B12

See reference [6].

trophic) contaminants presumably growing on the lysis products. Reinoculation of methanol liquid media with lysed colony material frequently resulted in a negative growth response. We therefore set out to use alternative procedures, instead of the conventional plating technique for the isolation of pure cultures. Repeated transfer of exponentially growing cell samples from one methanol liquid culture to the other remained possible and resulted in good growth. After each transfer the ability of the cells to grow on methanol agar was tested and this finally resulted in the isolation of variants which did not lyse readily on plating media, e.g. strain *Bacillus* sp. C1. A second successful method was a stepwise increase of temperature during growth of a mixed culture in methanol-limited chemostats. At temperatures around 60 °C the number of contaminating species gradually diminished and at 62 °C this finally resulted in the isolation of for instance strain *Bacillus* sp. PB1 in pure culture. The latter procedure was not always successful, i.e. one or two contaminants were found to remain present occasionally even at the higher temperatures, although at this point one *Bacillus* species had become dominant in the population. This opened up the possibility of applying a serial dilution technique in methanol liquid medium. After each round the highest dilution showing positive growth on methanol was subjected to a further dilution series. This procedure was combined with tests for good growth on methanol agar and thus resulted in the isolation of several pure cultures (e.g. strain *Bacillus* sp. AR2). When using the above techniques in various combinations, pure cultures of 6 methanol-utilizing *Bacillus* strains were obtained [6]. Al-Awadhi et al. [8,9], using a continuous enrichment technique (stepwise temperature increase), reported the isolation of a further 8 thermotolerant methylotrophic *Bacillus* strains with similar properties.

4. GENERAL PROPERTIES OF METHYLOTROPHIC *BACILLUS* STRAINS

The *Bacillus* strains obtained have many properties in common (Table 2). All strains are Gram-positive, rod-shaped bacteria with a strictly respiratory metabolism. Initially, all strains produced subterminal-central, ellipsoidal spores in swollen sporangia. Some strains lost this ability during subsequent maintenance. There is a clear correlation between loss of sporulation and good growth on methanol plates [6]. Maximum growth temperatures of 60 °C, or slightly higher, were observed. The optimum temperature for growth, however, is 50–55 °C. The isolates are therefore considered to be thermotolerant strains [6]. Their taxonomic position was studied by 16 S rRNA sequencing, DNA-DNA hybridization (in collaboration with W. Ludwig, G. Kirchhof and K.H. Schleifer, Munich), and by a phenotypic analysis (in collaboration with R.J. Sharp and D. White, Porton Down). The results indicate that they comprise at least one new *Bacillus* species, most closely related to *Bacillus firmus* (Arfman et al., unpublished).

A further interesting feature of the methylotrophic *Bacillus* strains is their marked resistance to high methanol concentrations. With *Bacillus* sp. C1 only a 50% reduction in growth rate is observed at a methanol concentration of 1.5 M. In addition to methanol, most of the strains showed good growth in mineral medium with maltose, mannitol, glucose or pyruvate, and in a variety of complex media.

The molar growth yields on methanol, measured at the optimum growth temperatures in methanol-limited chemostats, are among the highest reported for methylotrophic bacteria (Table 2). Enzyme analysis [10] revealed that carbon assimilation proceeds via the RuMP cycle of formaldehyde fixation, with the FBP aldolase cleavage and TK/TA rearrangement variants (Table 3). All isolates possessed high levels of hexulose-6-phosphate synthase, an enzyme specific for the RuMP cycle of formaldehyde fixation. Dissimilation of methanol-carbon occurred via the dissimilatory RuMP cycle. Methanol-grown cells oxidized methanol at a very high rate. No activity of PQQ-dependent methanol dehydrogenase (characteristic for the Gram-negative methanol-utilizing bacteria) was detected. Instead, high activities of an NAD-dependent methanol dehydrogenase (MDH) were found in all isolates. The high growth yields of the

Table 3

Specific activities (nmol/min per mg protein) of various enzymes in extracts of methanol-grown *Bacillus* sp. C1 and *Bacillus* sp. TS4

Enzyme	Activity in:	
	Bacillus sp. C1	*Bacillus* sp. TS4
Methanol oxidation rate (whole cells)	1100–1500 (75) [a]	1100–1500
1) Formaldehyde reductase	1220 (680) [a]	1265
Formaldehyde dehydrogenase	nd	nd
Formate dehydrogenase	nd	nd
2) Hexulose-6-phosphate synthase	17300 (515) [a]	19400
3) Hexulose-6-phosphate isomerase	5950	6700
4) Glucose-6-phosphate isomerase	1415	1480
5) Glucose-6-phosphate dehydrogenase (NADP)	475 (135) [a]	930
6) 6-Phosphogluconate dehydrogenase (NADP)	385	670
7) 6-Phosphofructokinase	600	670
8) Fructose-1,6-bisphosphate aldolase	370	1190
9) Transketolase	710 (460) [a]	1110 (140) [a]
10) Transaldolase	60 (9) [a]	140 (60) [a]
11) Phosphoriboisomerase	80	105
12) Ribulose phosphate 3-epimerase	775	650
KDPG aldolase/6-PG dehydratase	nd	nd
NADH oxidase	630	590
NADPH oxidase	25	30
NADH dehydrogenase	740	510

[a] Glucose grown cells; nd, not detectable; KDPG, 2-keto 3-deoxy 6-phosphogluconate; 6-PG, 6-phosphogluconate. See reference [10].

Bacillus strains on methanol thus appear to be based on the involvement of an NAD-dependent MDH, and the RuMP cycle, which is energetically the most efficient C_1-assimilatory pathway.

Growth in continuous culture with medium containing excess methanol resulted in the dissociation of the assimilation and dissimilation pathways. The excess methanol-carbon was fluxed into acetate [11,12].

5. PROPERTIES OF NAD-MDH

In *Bacillus* sp. C1 grown under methanol limitation at low dilution rate, NAD-MDH constitutes 20–30% of total soluble protein. The enzyme is NAD-specific and partially purified preparations catalyze the oxidation of various primary alcohols at the following relative rates: methanol, 44%; ethanol, 100%; n-propanol, 71%; n-butanol, 87%. In extracts and partially purified preparations the enzyme displayed biphasic kinetics towards methanol (apparent K_m values of 3.8 and 166 mM) and ethanol (apparent K_m values 7.5 and 70 mM). Biphasic kinetics was also observed towards NAD (apparent K_m values 15 and 190 μM), but not for formaldehyde (apparent K_m value 2.0 mM) in the reverse reaction. The enzyme has been purified to homogeneity and further characterized [10]. It is a decamer of 430000, composed of identical 43-kDa subunits. N-terminal sequence analysis (in collaboration with J.J. van Beeumen, Gent) revealed homology with Fe^{2+}-containing ADH2 from *Zymomonas mobilis* and ADH4 of *Saccharomyces cerevisiae*. The purified enzyme was almost completely devoid of NAD-MDH activity, whereas it was still able to catalyze the reverse, formaldehyde reductase, reaction. However, dehydrogenase activity could effectively be restored by adding a 50-kDa gel filtration fraction of crude extract and Mg^{2+} to the assay system. Further purification of the activator

protein resulted in the isolation of a 50-kDa protein consisting of two identical subunits of 25 kDa. No alcohol dehydrogenase, NADH dehydrogenase, acetaldehyde dehydrogenase, or hexulosephosphate synthase activity was associated with this protein. The kinetic properties of this system will be studied in further detail.

6. CONCLUDING REMARKS

Methylotrophic *Bacillus* strains are wide-spread and ubiquitous in nature. In view of the problems experienced in obtaining pure cultures it is not surprising that they have remained undetected for a long time. Most of the pure cultures obtained, however, grow rapidly and reproducibly on solid and liquid simple salts media. All strains investigated were found to possess a novel NAD-dependent methanol dehydrogenase with a relatively low affinity for methanol. The organisms nevertheless maintain a high turnover rate of methanol into formaldehyde (required for fast growth on the C_1 compound methanol) by synthesizing high levels of the enzyme. In addition, the high hexulose-6-phosphate synthase as well as NADH dehydrogenase activities observed in these bacteria may well ensure that, in vivo, the methanol dehydrogenase reaction is favoured over the formaldehyde reductase reaction. The methanol dehydrogenase present in *Bacillus* sp. C1 in fact is (part of) a rather complex system. On the basis of various observations it is concluded that the additional presence of a 50-kDa protein is required to maintain alcohol dehydrogenase activity.

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